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Laure Peyta[1], Kathleen Jarnouen[2,3], Michelle Pinault [1], Cedric Coulouarn[2,3], Cyrille Guimaraes[1], Caroline Goupille[1,5], Jean-Paul Pais de Barros[4], Stephan Chevalier[1], Jean-François Dumas[1], François Maillot[1,5], Grant M. Hatch[6], Pascal Loyer[2,3] and Stephane Servais[1].

[1]Inserm UMR1069, *Nutrition, Croissance et Cancer*, Université François Rabelais de Tours- 10, bd Tonnellé - 37032 Tours Cedex, France. *laure.peyta@etu.univ-tours.fr; michelle.pinault@univ-tours.fr; cyrille.guimaraes@gmail.com; caroline.goupille@univ-tours.fr, stephan.chevalier@univ-tours.fr; jean-francois.dumas@univ-tours.fr; maillot@med.univ-tours.fr; stephane.servais@univ-tours.fr*

[2]Inserm UMR S-991, *Foie, Métabolismes et Cancer*, CHU Pontchaillou, 2 rue Henri Le Guilloux, 35033 Rennes, France. *kathleen.jarnouen@univ-rennes1.fr; cedric.coulouarn@univ-rennes1.fr; pascal.loyer@univ-rennes1.fr*

[3]Université de Rennes 1, 2 rue du Thabor CS46510, 35065 Rennes cedex, France.

[4]Plateforme de Lipidomique. INSERM UMR866 / LabEx LipSTIC, 15 Bd Mal de Lattre de Tassigny, 21000 Dijon, France. *jppais@u-bourgogne.fr*

[5]CHRU de Tours, Département de Médecine Interne, 2, boulevard Tonnellé - 37044 Tours cedex 9, France.

[6]Department of Pharmacology and Therapeutics, Biochemistry and Medical Genetics, Faculty of Health Sciences, Center for Research and Treatment of Atherosclerosis, DREAM Children's Hospital Research Institute of Manitoba, University of Manitoba, 513 – 715 McDermot Avenue Winnipeg MB R3E 3P4, Manitoba, Canada. *ghatch@chrim.ca*

[*]Corresponding author:

Stephane SERVAIS, Laboratoire Nutrition, Croissance et Cancer - Inserm UMR1069

Faculté de Médecine - 10, bd Tonnellé - 37032 Tours Cedex

Email stephane.servais@univ-tours.fr; Phone +33 2.47.36.60.59; Fax +33 2 47 36 62 26

Running title: cardiolipin metabolism in cancer cachexia

Abbreviations: ALCAT1: acyl-CoA:lysocardiolipin acyltransferase; CDP-DAG: diphosphate-1,2-diacyl-sn-glycerol; CDS:cytidine-5'-diphosphate-1,2-diacyl-sn-glycerol synthase; CL: cardiolipin; CLS: cardiolipin synthase; DMSO: dimethylsulfoxide; ETS: electron transfert system; FCCP: Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; Hsc70: Heat shock cognate 70 kDa; iPLA2γ:calcium independent phospholipase A2 gamma; MLCL AT-1: monolysocardiolipin acyltransferase-1; MLCL: monolysocardiolipin; PC: phosphatidylcholine; PCC: peritoneal carcinosis induced cachexia; PE: phosphatidylethanolamine; PF: pair fed; PG: phosphatidylglycerol; PGC-1α: peroxisome proliferator-activated receptor gamma coactivator 1-alpha; PGP: phosphatidylglycerol phosphate; PGPP: phosphatidylglycerol phosphate phosphatase; PGPS: phosphatidylglycerol phosphate synthase; PI: phosphatidylinositol; PS: phosphatidylserine; SM: sphyngomyelin; TAZ: tafazzin; TNFα: tumor necrosis factor alpha; VDAC: voltage-dependent anion channel.

Abstract

Cardiolipin (CL) content accumulation leads to an increase in energy wasting in liver mitochondria in a rat model of cancer cachexia in which tumor necrosis factor alpha (TNFα) is highly expressed. In this study we investigated the mechanisms involved in liver mitochondria CL accumulation in cancer cachexia and examined if TNFα was involved in this process leading to mitochondrial bioenergetics alterations. We studied gene, protein expression and activity of the main enzymes involved in CL metabolism in liver mitochondria from a rat model of cancer cachexia and in HepaRG hepatocyte-like cells exposed to 20 ng/ml of TNFα for 12 h. Phosphatidylglycerolphosphate synthase (PGPS) gene expression was increased 2.3-fold ($p<0.02$) and cardiolipin synthase (CLS) activity decreased 44% ($p<0.03$) in cachectic rat livers compared to controls. CL remodeling enzymes monolysocardiolipin acyltransferase (MLCL AT-1) activity and tafazzin (TAZ) gene expression were increased 30% ($p<0.01$) and 50% ($p<0.02$), respectively, in cachectic rat livers compared to controls. Incubation of hepatocytes with TNFα increased CL content 15% ($p<0.05$), mitochondrial oxygen consumption 33% ($p<0.05$), PGPS gene expression 44% ($p<0.05$) and MLCL AT-1 activity 20% ($p<0.05$) compared to controls. These above findings strongly suggest that in cancer cachexia, TNFα induces a higher energy wasting in liver mitochondria by increasing CL content *via* upregulation of PGPS expression.

Highlights:

- First demonstration of liver cardiolipin metabolism alteration in cancer induced cachexia
- Direct implication of TNFα in mitochondrial energy wasting via cardiolipin modulation
- Increasing knowledge on cellular mechanism of liver energetic alteration during cancer cachexia

Key words: cardiolipin biosynthesis, cytokines, mitochondria, energy wasting, liver, cardiolipin remodeling, inflammation.

INTRODUCTION

Cancer cachexia is a multifactorial syndrome characterized by a progressive deterioration of nutritional status due to whole body negative energy balance driven by a reduced calorie intake (anorexia) and/or inappropriate energy expenditure (hypermetabolism) and systemic inflammation [1]. Depending on tumor type, cancer cachexia occurs in 30 to 80% of patients [2–4]. The negative energy balance induces white adipose tissue and skeletal muscle wasting which both impair quality of life and increase chemo- and radiotherapy associated toxicity in patients [5]. The mechanisms of cancer cachexia have not been fully elucidated and this explains the difficulties in managing therapy and restoring nutritional status in cancer patients. We recently investigated mechanisms involved in the hypermetabolism observed during cachexia in a rat preclinical model peritoneal carcinosis [6]. We demonstrated that peritoneal carcinosis-induced cachexia was associated with bioenergetic alterations in the healthy liver. Liver mitochondria from cachectic rats displayed significant increases in energy wasting and this was associated with reduced ATP production yield. Interestingly, liver mitochondria cardiolipin (CL) content was increased in cachectic rats in comparison to healthy controls [7]. Importantly, CL content was positively correlated with this increase in energy wasting. We subsequently demonstrated *in vitro* that CL content was indeed directly involved in the increase in liver mitochondria energy wasting [8]. Cancer cachexia not only affected the CL content but also CL fatty acid composition in liver mitochondria [6]. The mechanisms driving such liver mitochondrial CL alterations in cancer cachexia have not been investigated.

CL [bis-(1,2-diacyl-sn-glycero-3-phospho)-1′-3′-*sn*-glycerol] is a major and specific phospholipid of the mitochondria and on the inner membrane CL is required for the activity of a number of key mitochondrial enzymes involved in cellular energy metabolism [9,10]. CL metabolism is complex and includes both *de novo* biosynthesis and remodeling. In mammals, the first step of CL *de novo* synthesis is catalyzed by cytidine-5'-diphosphate-1,2-diacyl-*sn*-glycerol synthase (CDS) producing diphosphate-1,2-diacyl-*sn*-glycerol (CDP-DAG). Phosphatidylglycerol phosphate synthase (PGPS), the committed step of CL synthesis, catalyzes the formation of phosphatidylglycerol phosphate (PGP) from CDP-DAG. PGP is then dephosphorylated to phosphatidylglycerol (PG) by PGP phosphatase (PGPP) [11]. The final step is catalyzed by CL synthase (CLS) [12]. At this step CL is considered as nascent and will undergo

maturation by fatty acyl remodeling. Remodeling allows the formation of mature CL with defined fatty acyl composition. Initially deacylation by calcium independent phospholipase A_2 (iPLA$_2\gamma$) as well as other putative phospholipases [13] produces monolysocardiolipin (MLCL), this step is followed by resynthesis catalyzed by at least 3 enzymes: monolysocardiolipin acyltransferase-1 (MLCL AT-1), acyl-CoA:lysocardiolipin acyltransferase (ALCAT1) or the transacylase tafazzin (TAZ) [14–16].

Whereas there is a relative consensus about CL *de novo* synthesis in yeast and lower eukaryotes, regulation of the CL metabolic enzymes in higher eukaryotes is still poorly understood [17]. Previous studies in heart and myoblasts indicated that CL metabolism was upregulated by peroxisome proliferator-activated receptor α agonist [18] and peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) [19]. Since inflammation is a hallmark of cancer cachexia [20], we hypothesized that inflammatory cytokines may play a significant role in the dysregulation of CL metabolism in cancer cachexia and energy wasting increase in liver mitochondria. Our previous study clearly highlighted TNFα as the major cytokine (14-fold elevated serum concentration) in our preclinical model of cancer cachexia [6].

The aim of the present study was to investigate the mechanisms responsible for liver mitochondrial CL accumulation and its altered fatty acid composition in cancer cachexia. We examined gene expression, protein expression and activity of the main enzymes involved in CL biosynthesis and remodeling in liver mitochondria from a preclinical model of cancer cachexia [6]. In addition, we investigated the *in vitro* effect of the proinflammatory cytokine TNFα on CL metabolism related enzymes in the human hepatoma HepaRG hepatocyte-like cells that retain many characteristics of primary human hepatocytes [21].

MATERIALS AND METHODS

Materials

Undifferentiated HepaRG cells (BioPredict International France, Batch number HPR101007-A), [^{14}C]Glycerol-3-phosphate, [5-^{3}H]Cytidine 5'-riphosphate, [1-^{14}C]palmitoyl-phosphatidylcholine, [1-^{14}C]linoleoyl-Coenzyme A were obtained from Dupont, Mississauga, Ontario, Canada or Amersham, Oakville, Ontario, Canada. [^{14}C]PG was synthesized from [^{14}C]glycerol-3-phosphate as described [22].

Animal model

Liver from cachectic (peritoneal carcinosis induced cachexia, PCC) and healthy (pair fed, PF) rats were obtained as previously described [6].

Cell culture and treatments for in vitro inflammation study

As previously described [21] HepaRG cells line were cultivated in William's E medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin, 5 µg/ml insulin, and 50 µM hydrocortisone hemisuccinate. After 2 weeks, the medium was supplemented with 2% dimethyl sulfoxide (DMSO) and the cells were cultured for 2 more weeks. HepaRG hepatocytic cells from DMSO treated cultures were selectively detached using gentle trypsination and seeded at high density for cytokine treatments. Cells were incubated for 12 h with 20 ng/ml of TNFα.

Generation of SiPGPS HepaRG Cells

The validated siRNA (sc-94116, Santa Cruz Biotechnology) was used for knocking down the expression of PGPS in HepaRG hepatocyte-like cells. The siRNA (s1227, Ambion®) targeting albumin was used as control. Electroporation was performed using the Neon® transfection system (Life Technologies) available at the SynNanoVect core facility (Biogenouest, Rennes, France) as previously described [23]. Briefly, differentiated HepaRG hepatocyte-like cells were detached by trypsin incubation, then, 10^6 cells were electroporated with 40 picomoles of siRNA and the following parameters: one pulse of 1500 volts during 20ms. Cells were immediately seeded at the density of $2.5x10^5$ cells/cm2 and cultured in William's

E medium supplemented with 2% DMSO. Twenty four hours after electroporation, medium was discarded and cells were cultured in William's E medium without fetal calf serum and DMSO for 24h prior stimulation with 20 ng/mL of TNFα for 12h.

Transmission electron microscopy for mitochondrial content determination

HepaRG cells and liver biopsies were fixed by incubation for 24 h in 4% paraformaldehyde, 1% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2). Samples were then washed in phosphate-buffered saline (PBS) and post-fixed by incubation with 2% osmium tetroxide for 1 h. Samples were then fully dehydrated in a graded series of ethanol solutions followed by a propylene oxide bath. Pre-impregnation step was made by a propylene oxyde/Epon resin mixture (Sigma) and finally overnight in pure resin for impregnation of the samples. Cells were then embedded in Epon resin (Sigma), which was allowed to polymerize for 48 h at 60°C. Ultra-thin sections (90 nm) of these blocks were obtained with a Leica EM UC7 ultramicrotome (Wetzlar, Germany). Sections were deposited on gold grids and stained with 2% uranyl acetate, 5% lead citrate. Observations were made with a JEOL 1011 transmission electron microscope. Images were analysed using ImageJ software (NIH)

Phospholipid classes quantitation

Mitochondria were extracted from HepaRG cells by differential centrifugation. Then mitochondrial lipids were extracted following the protocol of Bligh and Dyer [24]. In order to quantify mitochondrial phospholipids (cardiolipin [CL], phosphatidylethanolamine [PE], phosphatidylinositol [PI], phosphatidylcholine [PC], phosphatidylserine [PS] and sphyngomyelin [SM]), standards and samples were loaded on silica plates using Linomat V sample applicator (CAMAG, Muttenz Switzerland). Solvant consisted of chloroform, acetone, acetic acid methanol and water (6/8/2/2/1 v/v/v/v/v) modified from [25]. After migration, the plates were immersed into the following solution (10% wt/vol) copper sulfate in 8% (vol/vol) phosphoric acid solution) and then heated at 160°C for 15 min to stain all of the phospholipids. Quantification was performed using Thin Layer Chromatography-visualizer Reprostar 3® and WinCats

VideoScan® software (CAMAG, Muttenz Switzerland). Results were expressed in percent of total mitochondrial phospholipids.

CL fatty acid molecular species Analysis by LC-MS²

Lipids were extracted from cell pellets (6-8 millions) according to the method of Folch *et al*. [26].

In brief, cells were spiked with (14:0)4-CL used as internal standard (250 ng, Avanti Polar Lipids, Coger, Paris, France). Lipids were extracted with 1 mL of saline and 3.5 mL of CHCl3/MeOH (2/1) for 10 min followed by addition of 1.25 mL CHCl3 for 10 min and finally 1.25 mL of distilled water for 5 min. Organic phase was collected and dried under vacuum. Lipid extracts were further solubilized in 100 µL of CHCl3/MeOH/H2O (60/30/4.5). LCMS/MS analyses were performed with a 1200 series HPLC system coupled to a 6460 triple quadrupole mass spectrometer equiped with a JetStream electrospray ionization source (Agilent Technologies). HPLC separation was achieved with a Zorbax EclipsePlus C18 - 2.1 mm x 100 mm, 1.8 µm column (Agilent Technologies) maintained at 50°C. The mobile phases consisted of ACN, water, 28% NH3 in water and acetic acid (90/10/0.2/0.5 v/v/v/v) (A) and of IPA, water, 28% NH3 in water and acetic acid (90/10/0.2/0.5 v/v/v/v) (B). The linear gradient was as follows: 50% B for 5 min, up to 80% B in 10 min, up to 100% in 15 min and maintained at 100 % for 5 min. Column was equilibrated with 50% B for 5 min before each injection. The flow rate was set up at 0.4 mL/min. Four microliters were injected onto the LCMS/MS system. Analyses were performed in negative ion mode by Selected Reaction Monitoring (SRM) as previously described [27].

RNA isolation and real time PCR assay

Total RNA from livers or HepaRG cells were extracted with RNeasy Mini kit (QIAGEN; Cat N° 74106) following manufacturer's instructions. Reverse transcription was performed using 1µg of total ARN with the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Part N° 4368813). RNA levels of various genes were determined using Taqman- (HepaRG cells) or SYBR green (rat liver)-based quantitative PCR (qPCR) technology performed using Fast Real-time 7900HT system (Applied Biosystems). The 18S and GAPDH were used as housekeeping genes for rat livers and HepaRG cells, respectively. Data were quantified with the StepOne Plus software v2.2.1 and the results were expressed as relative to pair-fed animals or untreated HepaRG cultures arbitrarily set to 1. Primer sequences: rat 18S

5'-CGGCTACCACATCCAAGGAA -3' and 5'- GCTGGAATTACCGCGGCT-3', rat CDS1 5'-GACTCCAGAACACTGTATCT-3' and 5'- GCTCACAGCAGCAATTTGAT-3', rat PGPS 5'-GGCAGATAAAAATAGCCAAG-3' and 5'- CAATCCACCAGTTCCTGTTCC-3', rat CLS 5'-GAACACTAGCTAAGTACTTC-3' and 5'- ACTGCTGTATTGACCTTGC-3', rat TAZ 5'-CTGAAGTTGATGCGTTGGACC-3' and 5'- TGTACCCAGTCCCCATGGTT-3'. Taqman primers for human GAPDH Hs02758991_g1, human PGPP Hs00864157_g1, human PGPS Hs00375485_m1, human CLS Hs00219512_m1, human MLCL AT-1 Hs00426191_m1, human TAZ Hs00794094_m1,human human ALB (Hs00910225_m1) and human TATA Binding Protein (TBP) (Hs00427620_m1).

Western blotting

Rat liver and HepaRG cell mitochondria were isolated as described [28]. 50 μg of mitochondrial protein were separated on 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred onto a polyvinylidene fluoride membrane, and probed with antibodies against CLS (14845-1-AP, Proteintech, Manchester, UK), Heat shock cognate 70 (Hsc70, SC7298, SantaCruz) and Voltage Dependent anion channels (VDAC) (AB14734 Abcam) (loading control) followed by horseradish peroxidase-coupled detection (Pierce ECL Western Blotting Substrate). Secondary antibodies were obtained from SantaCruz. The protein band intensities were analyzed by densitometry (MF-ChemiBIS 3.2, DNR Bioimaging Systems, MultiGauge software, Fujifilm).

Assay of enzyme activities

Rat liver and HepaRG cell mitochondria were isolated as described [28]. CDS, PGPS enzyme activities were determined as described [22]. CL synthase activity was assayed exactly as described by Schlame and Hostetler [29], except that the assay contained 0.05-0.1 mg of protein, the pH of the assay was 8.5 and the samples were sonicated for 10 s in a Branson model 1200 sonicator before incubation. Incubation was at 37°C for 60 min with [14C]PG (sp.radioactivity 45000 d.p.m./nmol). MLCL AT-1 activity was determined as described [30].

High-resolution respirometry

High resolution respirometry was performed using a 2-ml chamber OROBOROS® Oxygraph 2K (Oroboros Instruments, Innsbruck, Austria) at 37°C. Respiration rates were calculated as the time derivative of oxygen concentration measured in the closed respirometer and expressed per million viable cells and corrected by non-mitochondrial oxygen consumption (antimycin A). Intact cells (0.5×10^6 cells per milliliter) were analyzed in their respective energy substrate-containing cultivation medium: ROUTINE respiration (no additions), LEAK respiration (oligomycin-inhibited, 8 µg/mL), and ETS (Electron Transfert System) capacity (maximum uncoupled respiration) induced by Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP, 0.8 mM). Oxygen consumption (O_2) was also measured on permeabilized cells (digitonin 11µg/ml) in respiration buffer (10 mM KH_2PO_4, 300 mM mannitol, 10 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA and 1 mg/ml BSA fatty acid free) at final cell density of 0.5×10^6 cells per milliliter. Pyruvate, malate and succinate were used as respiratory substrates.

Statistical analysis

Data are expressed as means ± S.E.M. Statistical analyses were performed using GraphPad Prism®. The differences between two groups were evaluated by non parametric Mann Whitney *t*-test. Values showing $p < 0.05$ were considered statistically significant unless otherwise indicated.

RESULTS

Mitochondrial content is unchanged during cancer cachexia

CL is a unique phospholipid only found in mitochondrial membranes. In order to exclude that mitochondrial biogenesis was the origin for CL accumulation (+50%), we first quantified liver mitochondrial mass from PF and PCC rats using electron microscopy. Our results did not show any difference between the two groups of rats (Figure 1). Indeed, hepatocytes from PF rats contained 20.32% +/- 1.95 of mitochondria whereas 22.24% +/- 1.58 for PCC rat hepatocytes.

Liver PGPS and CLS are altered in cancer cachexia

To investigate the biochemical alterations in CL biosynthesis during cancer cachexia four enzymes of CL metabolism were studied: CDS-1, PGPS, PGPP, and CLS in liver of healthy (PF) and cachectic rats (PCC). CDS-1 mRNA level and CDS enzyme activity were similar between PCC and PF rats (Figure 2A and B). In contrast, gene expression of PGPS was significantly increased by 2.3-fold ($p = 0.02$) in liver of PCC compared to PF rats (Figure 2C). In addition, PGPS activity appeared to be increased by 30% in PCC compared to PF rats (Figure 2D) without reaching statistical significance. PGPP mRNA expression was unaltered between PCC and PF rats (0.56 +/- 0.20 and 0.63 +/- 0.14, respectively). CLS mRNA and protein expression were similar between PCC and PF rats (Figure 3 A and B). However, mitochondrial CLS enzyme activity was significantly decreased by 44% in PCC rats ($p = 0.03$) compared to PF rats (Figure 3 C).

Liver CL remodeling processes are affected by cancer cachexia

We previously reported important modifications in CL fatty acid composition in liver mitochondria from cachectic rats [7] suggesting a modulation in CL remodeling. We examined four enzymes implicated in this process: iPLA2γ, MLCL AT-1, ALCAT1 and TAZ in livers of healthy (PF) and cachectic rats (PCC). iPLA2γ enzyme activity and mRNA expression were not different from PCC and PF rats (mRNA: 0.64 +/- 0.25 *vs* 0.88 +/- 0.30 and activity: 767 +/- 160 *vs* 823 +/- 46 $pmol.mg^{-1}.min^{-1}$, respectively). In addition

MLCL AT-1 enzyme activity was significantly increased by 30% in PCC rat liver mitochondria compared to PF rats (p = 0.01) (Figure 3 D). ALCAT1 mRNA expression was similar in liver between PCC and PF rats (1.24 +/- 0.28 *vs* 1.27 +/- 0.19, respectively). Finally, TAZ mRNA expression was significantly increased (50%) in PCC rats compared to PF rats (p = 0.02) (Figure 3 E).

TNFα treatment increases specifically hepatocyte CL content without changes in mitochondrial mass

Since TNFα was elevated 14-fold in plasma in rat model of peritoneal carcinosis induced cancer cachexia, the effect of exogenous TNFα addition to HepaRG cells on CL metabolism was examined. Incubation of HepaRG cells with TNFα resulted in a significant 15% ($p<0.05$) increase in CL content compared to controls (Figure 4 A). In contrast, PE, PI, PC+PS and SM content did not differ between control cells and TNFα treated cells. In order to confirm that CL accumulation in response to TNFα was not due to an increase in mitochondrial mass, we quantified mitochondria area and number per cell area using transmission electron microscopy (Figure 4 B, C and D). Our result clearly demonstrated that TNFα has no effect on mitochondrial content (Control: 10.12% +/- 0.33, TNFα: 10.26% +/- 0.30 mitochondrial area % of cell area).

TNFα treatment alters HepaRG mitochondrial bioenergetics

Since CL plays a role in cellular energy metabolism and CL content was elevated in HepaRG cells treated with TNFα, we examined oxygen consumption in these cells. In intact HepaRG cells TNFα induced an overall increase in O_2 consumption (Figure 5). In fact, under routine conditions (cells using their own substrates in an energetic state with ATP production as a function of the energy needs of the cells) O_2 consumption was 33% higher in cells incubated with TNFα compared to control cells (figure 5 A). In the non-phosphorylating state, O_2 consumption (reflecting energy leak) in the presence of oligomycin, was significantly increased by 57% in cells treated with TNFα compared to controls. TNFα induced a significant 53% increase in maximal O_2 consumption (uncoupled state, electron transport chain maximal capacities ETS) compared to controls (figure 5A). When cells were permeabilized, we observed globally the same effect of TNFα on mitochondrial bioenergetics using pyruvate, malate and succinate as

substrates (Figure 5 B). O_2 consumption in non-phosphorylating (state 4) was significantly increased 33% in TNFα-treated cells compared to control cells (figure 5B). In the phosphorylating state (state 3), TNFα treated cells had a 17% significant increase in O_2 consumption compared to controls. Finally, uncoupled state related- O_2 consumption was increased 14% with TNFα compared to controls, however, this did not reach statistical significance (figure 5 B).

TNFα treatment increases PGPS gene expression in HepaRG cells

We examined the mechanism for the increase in CL in TNFα-treated cells. Incubation of HepaRG cells with TNFα did not affect mRNA expression of PGPP or CLS compared to controls (Figure 6 A and C). In contrast, PGPS mRNA expression was significantly increased 44% in cells incubated with TNFα compared to controls (Figure 6 B). Interestingly, incubation of HepaRG cells with TNFα did not affect TAZ, MLCL AT-1 or ALCAT1 mRNA expression (Figure 6 D, E and F). In addition, incubation of HepaRG cells with TNFα did not affect enzyme activities of CDS, PGPS and CLS compared to controls (Figure 7 A, B and C). In contrast, MLCL AT-1 activity was significantly increased 20% in cells treated with TNFα compared to controls (Figure 7 D).

TNFα treatment alters CL fatty acid molecular species in HepaRG cells

Next we asked if TNFα induced increase in MLCL AT-1 activity was associated to modification in CL molecular species. In fact, CL molecular species $(18:1)_3$-(18:2) and $(18:2)_3$-(16:1), have significantly altered content, respectively +15% and -10%, in TNFα treated hepatocytes compared to controls (Figure 8). Furthermore (16:0)-$(18:1)_2$-(18:2) and $(18:1)_4$ content was increased by 17% and 20% in HepaRG cells incubated with TNFα, without reaching statistical significance (p= 0.08) (Figure 8).

TNFα increases CL content by PGPS upregulation in HepaRG cells

Next we tested the direct connection between TNFα, CL content and PGPS expression. PGPS expression in siPGPS HepaRG cells was significantly reduced by 63 % in comparison of siALB cells (0,37 +/- 0,07

vs 1.00 +/- 0.11, relative expression/TBP respectively, N=4, p=0.028). Moreover, CL content was not affected by TNFα in siPGPS cells compared to controls (figure 9).

DISCUSSION

The aim of the present study was to investigate the mechanisms responsible for liver mitochondrial CL accumulation and alteration of CL fatty acyl composition in a preclinical model of cancer cachexia. For this purpose, we examined gene and protein expression and activities of the main enzymes involved in CL biosynthesis and remodeling in liver mitochondria using a preclinical model of cancer cachexia. In addition, we investigated *in vitro* the effect of the proinflammatory cytokine TNFα, a hallmark of cancer cachexia, on CL content and the CL metabolic enzymes in HepaRG hepatocyte like cells.

We observed discordant regulation of CL biosynthetic and remodeling enzymes at both the activity and gene expression level in liver mitochondria of cachectic rats compared to controls. We found that gene expression of PGPS and TAZ were significantly increased and that enzyme activities of PGPS (statistical trend -30%) and MLCL AT-1 were increased and enzyme activity of CLS decreased in liver from cancer cachexia rats. PGPS catalyzes the committed step of CL biosynthesis [14]. The observed elevated mRNA expression and activity of PGPS in livers of PCC rats might increase precursor supply for CL *de novo* synthesis. A previous study showed increased liver CL pool size was associated with an increased expression and activity of PGPS during rat liver regeneration [31]. Moreover, the importance of PGPS in controlling CL level was previously demonstrated in Chinese hamster ovary (CHO) cells [32]. In that study, a temperature sensitive CHO cell line defective in PGPS exhibited reduced cellular levels of CL. CLS mRNA and protein expression were unaltered in liver of PCC rats compared to PF rats. Surprisingly, CLS enzyme activity was reduced approximately 50% in liver mitochondria of PCC rats compared to PF rats. The observed decrease in CLS activity could be a compensatory mechanism to counteract the increased CL formed in liver of PCC rats. We previously showed that CLS mRNA expression does not correlate with endogenous CLS enzyme activity [33]. In *Micrococcus lysodeikticus* the end-product of CL synthesis, CL, was shown to inhibit CLS at several concentrations [34]. In addition, in rat liver mitochondria exogenous addition of CL was shown to markedly inhibit CLS activity [35]. Since CL levels were elevated in liver mitochondria of cachectic rats this would explain why CLS enzyme activity was reduced. Thus, although CLS activity was decreased by a higher level of CL, the elevated

mRNA expression of PGPS could lead to an increase in precursor synthesis for increased CL *de novo* biosynthesis provoking CL accumulation in livers of cachectic rats. These data represent the first direct demonstration of dysregulation of CL biosynthesis in cancer cachexia.

We previously observed a lower n−6/n−3 polyunsaturated fatty acid ratio in CL in liver mitochondria prepared from cancer cachectic rats [7]. Liver CL is enriched with linoleic acid and this polyunsaturated fatty acid enrichment of CL can be achieved by a CL transacylase encoded by the *Taz* gene [36] or by transfer of linoleoyl-CoA to MLCL by MLCL AT-1 [37]. Gene expression of ALCAT1, an enzyme capable of acylating MLCL with polyunsaturated species of fatty acid to form polyunsaturated fatty acid enriched CL [15], was unaltered in liver of PCC rats compared to PF rats. In addition, the activity of iPLA2γ, an enzyme known to deacylate CL [13], was unaltered in PCC rats compared to PF rats. In contrast, we observed a significant increase in both MLCL AT-1 enzyme activity and in TAZ mRNA expression. This increase in TAZ expression and MLCL AT-1 enzyme activity in liver of PCC rats was likely responsible for the altered fatty acid profile of CL observed in liver cachexia compared to control animals [7].

Since inflammation is a hallmark of cancer cachexia [20], we investigated the potential role of inflammatory cytokines in CL related-mitochondrial bioenergetics alterations in HepaRG hepatocytes. We previously identified TNFα as a major cytokine in our preclinical model of cancer cachexia [6]. In addition, an increased expression of TNFα has been observed in liver of cancer cachectic rats [38]. Therefore we examined *in vitro* the effects of TNFα treatment on HepaRG cell CL metabolism. Incubation with TNFα for 12 h increased mitochondrial CL content by 15% in HepaRG cells without affecting the level of other phospholipids. This increase in CL content was not related to an increase in mitochondrial mass in the cells treated with TNFα. We subsequently explored whether this increase in CL content in HepaRG cell mitochondria, mediated by TNFα, was associated with modification in mitochondrial bioenergetics. TNFα-treatment resulted in a significant increase in oxygen consumption in different energy states. The effect of TNFα on mitochondrial bioenergetics is subject to debate. Previous studies reported that TNFα treated cells exhibited inhibition of respiratory chain activity leading to enhanced superoxide production and apoptosis [39–42]. In contrast, other studies have demonstrated an increase in

mitochondrial oxygen consumption in TNFα treated cells [43–45]. This discrepancy highlights the fact that TNFα-mediated effects are very much dependent on cell type, cytokine concentration and treatment duration making comparison between studies difficult. However, our results are in agreement with recently published data by Drabarek and collaborators [44] in that our experimental protocol closely resembled their experimental conditions (hepatocyte cells, short term exposure and low TNFα concentration). Since mitochondrial biogenesis markers PGC-1α and mitochondrial transcription factor A protein expression were unaffected by TNFα treatment (data not shown), the increase in oxygen consumption by 30-40% (depending of cell configuration) in the non-phosphorylating state was clearly an indication for an increase in energy wasting. Furthermore, we previously demonstrated that in cancer cachexia, CL content of rat liver biopsies are increased and positively correlated to energy wasting in mitochondria [7]. Moreover, we successfully reproduced *in vitro* this effect by mitochondrial membrane CL enrichment demonstrating a direct link between CL content and oxidative phosphorylation efficiency [8]. Taken together, all of these results indicate that TNFα may induce increase energy wasting in hepatocytes *in vivo* by increasing CL content.

Since *in vitro* treatment of HepaRG cells with TNFα increased CL content and energy wasting similar to what we previously observed in cachectic rat liver biopsies [7], we postulated that TNFα signaling regulated specifically PGPS gene expression in cancer cachexia and resulting in the increase in CL. Previously we showed that incubation of H9c2 cells with TNFα induced an increase in PGPS [46]. Thus, we investigated if TNFα regulated gene expression of PGPS and other CL metabolism related enzymes in HepaRG cells. A 12 h treatment of HepaRG cells with TNFα resulted in an increase in PGPS gene expression and an increase in MLCL AT-1 activity. These data are in agreement with previous published data demonstrating a TNFα induced-PGPS overexpression in cardiomyocyte cell line [46]. Moreover we had previously shown that MLCL AT-1 activity is regulated in concert with the level of CL and CL biosynthesis in rat heart [47]. The observed increase in MLCL AT-1 activity after treatment of HepaRG cells with TNFα is likely required to support elevated CL remodeling with increased CL synthesis. In support of this were the observed alterations in CL fatty acid molecular species composition.

Finally, the absence of modification in CL content in siRNA PGPS-transfected HepaRG cells exposed to TNFα highlighted that action of TNFα on CL content is mediated by PGPS.

In conclusion, this is the first study that demonstrates alteration in liver CL metabolism related enzymes in cancer cachexia. In addition, our data clearly demonstrate that TNFα treatment increases CL content in hepatocytes. These data strongly suggest that in cancer cachexia, TNFα is one of the factors responsible for inducing the increase in energy wasting in liver mitochondria by increasing CL content through upregulation of PGPS expression.

Acknowledgements

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Figure legends

Figure 1: Liver mitochondrial mass is unchanged during cancer cachexia. (A) mitochondrial area quantification with (B) and (C) example of electron microscopy image from liver of PF and PCC rat. Values represent the mean ± SEM. Pair-fed rats (PF, white bar) and peritoneal carcinosis induced cachexia rats (PCC, black bar) with N = 3 per group.

Figure 2: Liver PGPS gene expression is upregulated in cancer cachexia. (A) CDS1 relative expression normalized to 18S mRNA, (B) activity and (C) PGPS relative expression normalized to 18S mRNA (D) PGPS activity. Values represent the mean ± SEM. Pair-fed rats (PF, white bar) and peritoneal carcinosis induced cachexia rats (PCC, black bar) with N = 7 per group. * $p<0.05$ compared to PF.

Figure 3: Liver CLS activity is decreased whereas CL remodeling enzymes are upregulated in cachectic rats. (A) CLS relative expression normalized to 18S mRNA, (B) CLS protein expression normalized by Hsc70 protein expression, (C) CLS activity, (D) MLCL AT-1 liver activity and (E) TAZ relative expression normalized to 18S mRNA. Values represent the mean ± SEM. Pair-fed rats (PF, white bar) and peritoneal carcinosis induced cachexia rats (PCC, black bar) with N = 7 per group. * $p<0.05$ compared to PF.

Figure 4: TNFα treatment of HepaRG cells results in increased CL content without modification of mitochondrial mass. Cells were treated for 12 h with 20 ng/ml of TNFα. (A) Mitochondrial phospholipid content with Cardiolipin (CL), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylcholine and phosphatidylserine (PC+PS), and sphyngomyelin [SM]. Values represent the mean ± SEM (N = 6 per group). White bars: control treatment; black bars: TNFα treatment. * $p<0.05$, compared to control treatment. (B) Mitochondrial area in % of HepaRG area. (C) and (D) example of electronic microscopic images of HepaRG cells in control condition and TNFα treatment, respectively.

Figure 5: TNFα treatment of HepaRG cells results in increased O_2 consumption. Cells were treated for 12 h with 20 ng/ml of TNFα and then high resolution respirometry performed on (A) intact cells or on (B)

permeabilized cells using pyruvate, malate and succinate as substrate. Values represent the mean oxygen (O_2) consumption (pmol / min / 10^6 cells) ± SEM (N= 6 per group). White bars: control treatment; black bars: TNFα treatment. * p<0.05, ** p<0.01 compared to control treatment.

Figure 6: TNFα treatment results in increased PGPS gene expression in HepaRG cells. Gene expression of (A) PGPP, (B) PGPS, (C) CLS, (D) MLCL AT-1, (E) Tafazzin and (F) ALCAT1. Cells were treated for 12 h with 20 ng/ml of TNFα. Total RNA was isolated and mRNA expression of the above genes determined. Values are normalized by GAPDH expression. Values represent the mean ± SEM (N = 9 per group). White bars: control treatment; black bars: TNFα treatment. **** p<0.0001, compared to control treatment.

Figure 7: TNFα treatment of HepaRG cells increases MLCL AT-1 activity. HepaRG cells were treated for 12 h with 20 ng/ml of TNFα and activity of (A) CDS1, (B) PGPS, (C) CLS and (D) MLCL AT-1 determined. Values represent the mean ± SEM (N = 5 per group). White bars: control treatment; black bars: TNFα treatment. * p<0.05 compared to control cells.

Figure 8: TNFα treatment of HepaRG cells affects fatty acid molecular species composition. HepaRG cells were treated for 12 h with 20 ng/ml of TNFα and CL fatty acid molecular species were analyzed (in % of total CL molecular species). Values represent the mean ± SEM (N = 6 per group). White bars: control treatment; black bars: TNFα treatment. * p<0.05, ** p<0.01 compared to control treatment.

Figure 9: TNFα treatment does not affect CL content in HepaRG siPGPS cells. HepaRG cells (siPGPS) were treated for 12 h with 20 ng/ml of TNFα. Cardiolipin content (CL) in siPGPS HepaRG cells +/- TNFα. Values represent the mean ± SEM (gene expression: N = 4 per group; cardiolipin content: N=4 per group). White bars: control treatment; black bars: TNFα treatment.

Figures

Figure 1: Liver mitochondrial mass is unchanged during cancer cachexia

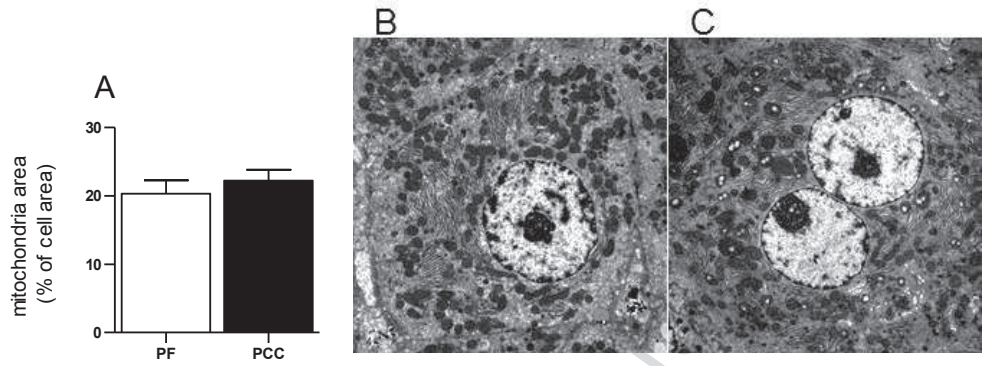
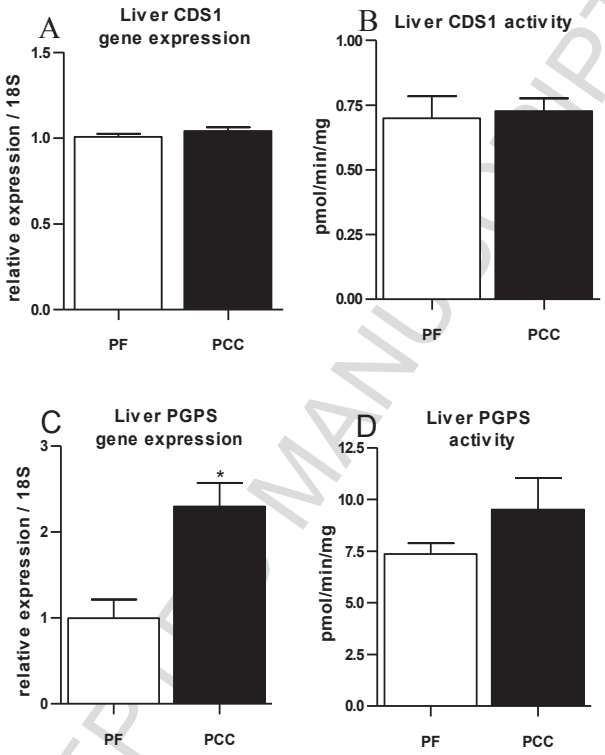


Figure 2: Liver PGPS gene expression is upregulated in cancer cachexia.



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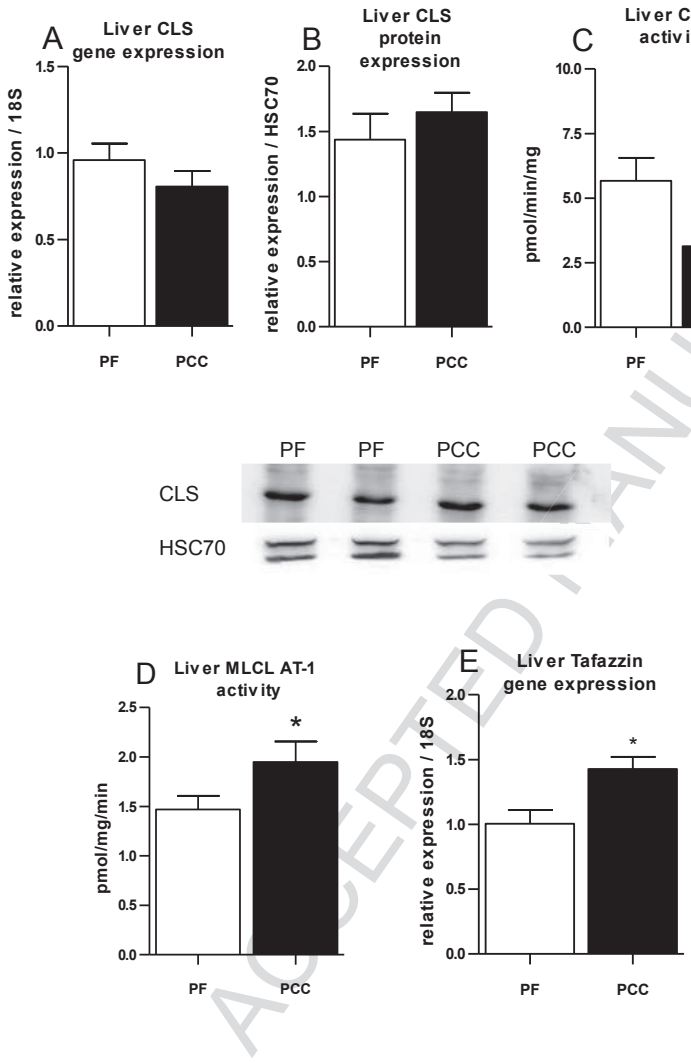
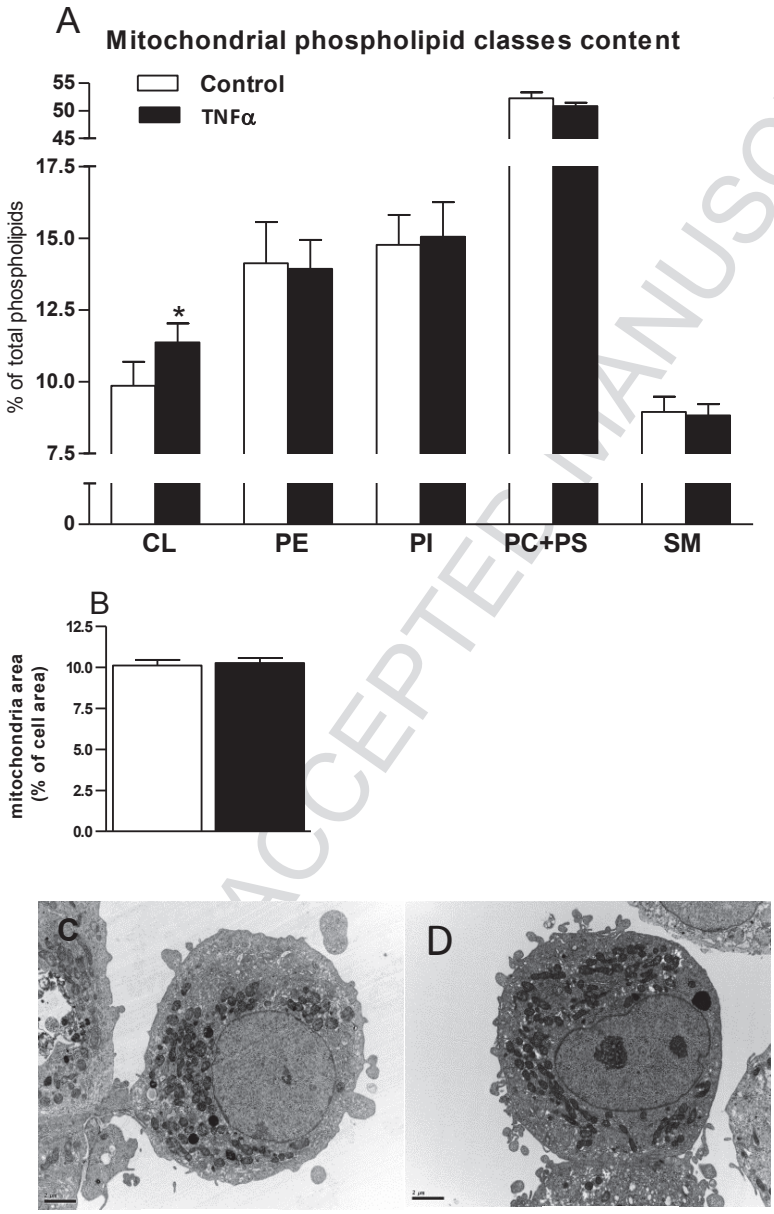


Figure 4: TNFα treatment of HepaRG cells results in increased CL content without modification of mitochondrial mass.



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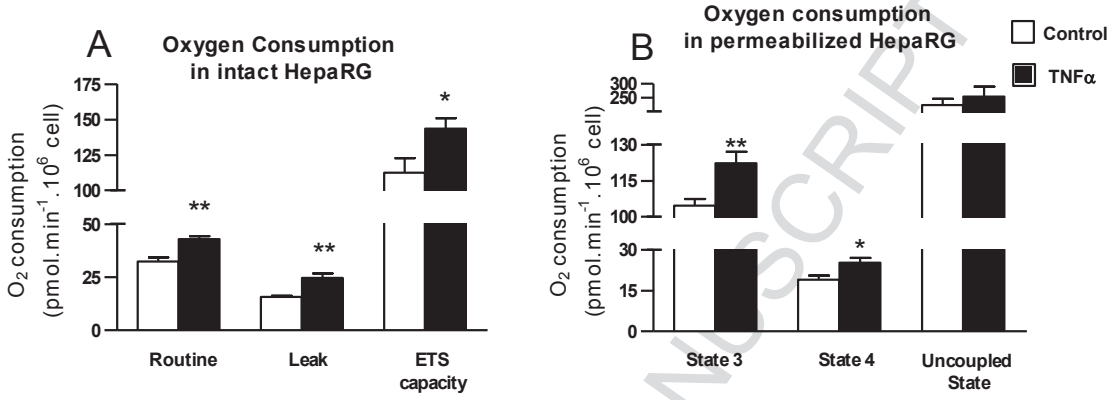


Figure 6: TNFα treatment results in increased PGPS gene expression in HepaRG cells.

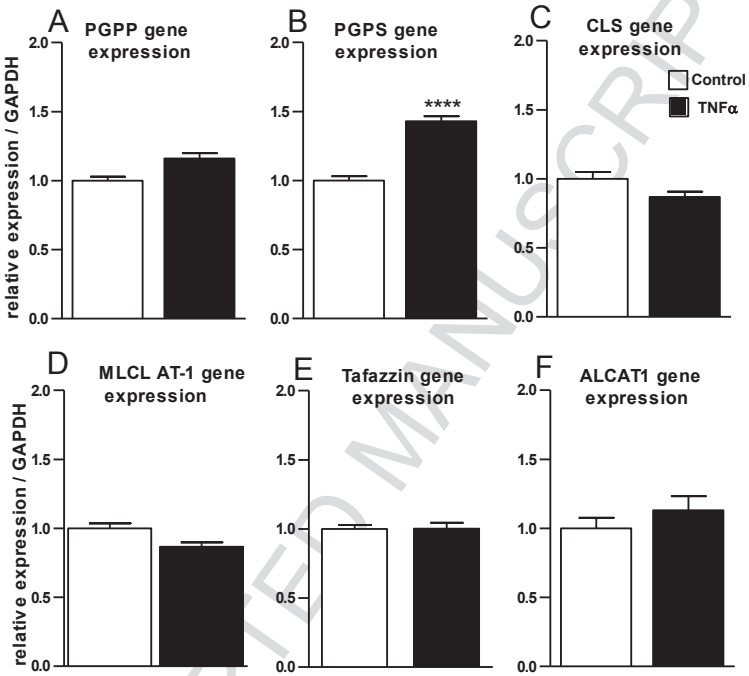


Figure 7: TNFα treatment of HepaRG cells increases MLCL AT-1 activity.

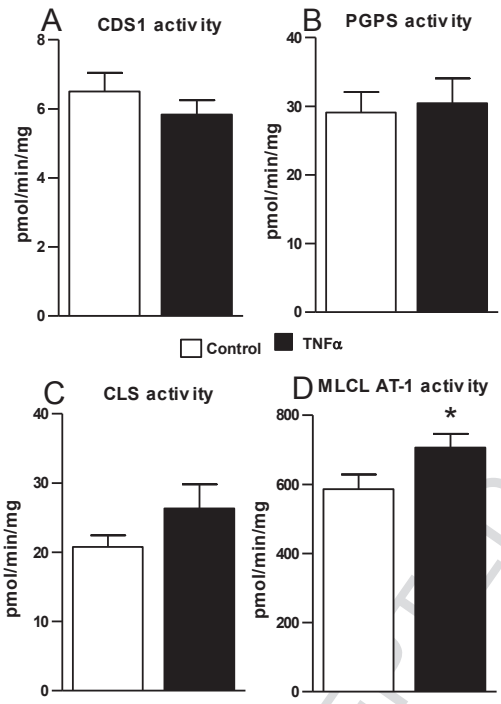


Figure 8: TNFα treatment of HepaRG cells affects fatty acid molecular species composition.

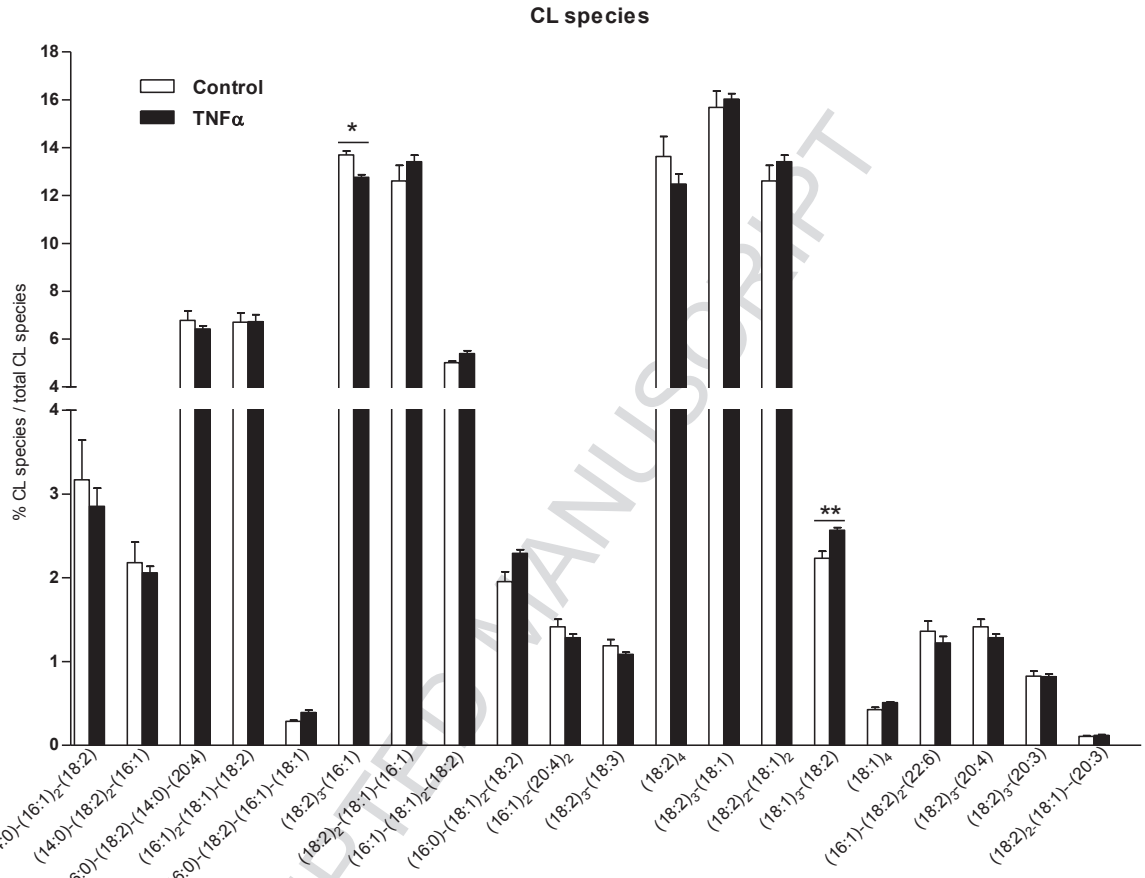
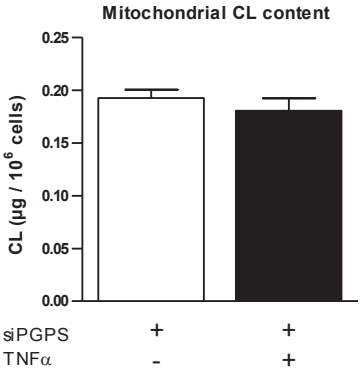


Figure 9: TNFα treatment does not affect CL content in HepaRG siPGPS cells



Conflict of interest

All the authors of the article « Regulation of hepatic cardiolipin metabolism by TNFα: implication in cancer cachexia" submitted to BBA- Molecular and Cell Biology of lipids, declare no conflict of interest.

Laure Peyta, Kathleen Jarnouen, Michelle Pinault, Cedric Coulouarn, Cyrille Guimaraes, Caroline Goupille, Jean-Paul Pais de Barros, Stephan Chevalier, Jean-François Dumas, François Maillot, Grant M. Hatch, Pascal Loyer and Stephane Servais have nothing to disclose.